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PRINCIPAL INVESTIGATOR: George F. Koob, Ph.D.
Eric P. Zorrilla, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, CA 92037

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George F. Koob, Ph.D.
Eric P. Zorrilla, Ph.D.

E-mail: gkoob@scripps.edu

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14. ABSTRACT
The work that constitutes this Final Addendum Report derived from the hypothesis that "individual differences in stress reactivity derived from selective breeding generalize to a behavioral phenotype that will reflect a predisposition to stress and negative affect." The results indicate that ACTH responsiveness to footshock stress is heritable, is a generalizable trait that also alters corticosterone responsiveness to other stressors, and is amenable to selective breeding in the rat. Genetic differences in stress responsiveness in replicate line 1 were associated with differences in anxiety-like behavior, body weight gain and voluntary intake of sweet solutions and ethanol -- possible indications of altered hedonic homeostasis. For some traits, the high stress responsive line was found to diverge from outbred stock, whereas for other traits the low stress responsive line was found to be different. The replicate lines may help identify the genetic basis of hyper- and hypo-stress responsiveness and associated stress-related disorders.

15. SUBJECT TERMS
Stress, anxiety, ethanol, alcoholism, obesity, animal model, selective breeding, genetics, rat

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INTRODUCTION:

Enduring dysregulation of stress response systems afflicts as many as one-third of combatants in major conflicts, and disorders of the stress response systems related to military operations result in substantial personal and public costs. The work that constituted this Addendum Report derived from our previous hypothesis "that chronic activation of the brain corticotropin releasing factor system can lead to permanent damage to brain monoamine systems and that individual differences in genetic background can exaggerate such dysregulation." The new hypothesis, based on an Alteration in Statement of Work, was that "individual differences in stress reactivity derived from selective breeding generalize to a behavioral phenotype that will reflect a predisposition to stress and negative affect."

To test this hypothesis, the following two Specific Aims were pursued: 1) To continue the successful breeding of replicate rats lines selected for hyper- and hypo-responsiveness of the hypothalamic pituitary-adrenal (HPA) axis to stress (HSR vs. LSR lines); and 2) To phenotype HSR and LSR sublines with respect to anxiety-, depressive- and other affective spectrum-like behaviors under baseline and social stress conditions. With respect to Specific Aim 1, Line 1 was in its seventh generation and Line 2 in its fifth generation at the onset of the Addendum. During the funding period, Line 1 advanced to the ninth generation and Line 2 to the seventh generation, representing 4 generations of progress. In addition, reference stock of N/NIH outbred rats were maintained for each replicate line. These rats provide controls for the genetic background of HSR and LSR rats and were bred in parallel for each replicate line, reflecting in total a further 4 generations of breeding. HSR and LSR rats continue to differ in adrenocorticotropin hormone (ACTH) responses to footshock stress. During the funding period, it was additionally shown that they also differ in downstream corticosterone responses to social stress, a different stressor than was used for selection. The results confirm the separation of HPA-axis responses of HSR and LSR lines and demonstrate their generalizability to effectors of the stress response (i.e., corticosterone) and to other stressors.

With respect to Specific Aim 2, rats were phenotyped on several endpoints putatively related to stress, including body weight, anxiety-like behavior, locomotor activity in novel or familiar environments, and intake of sweet or ethanol-containing solutions. Relative to LSR rats, HSR rats were found to be heavier from early adulthood (but not at weaning) through at least 1 year of age, to exhibit increased anxiety-like behavior in the shock-probe test, to be less active in novel environments,

to drink more of a palatable saccharin solution, and to drink more ethanol-containing solutions, especially following periods of forced abstinence. The results confirm that the selectively bred lines have diverged on many phenotypes thought to be related to stress. Thus, the HSR and LSR lines may represent a valuable model to identify the molecular basis for heritable vulnerability to chronic stress-related pathologies, such as anxiety, overweight, or alcohol use disorders. The lines also may be useful for studying potential prophylactic or therapeutic treatments for such disorders.

BODY:

Specific Aim 1: To continue the successful breeding of replicate rats lines selected for hyper- and hypo-responsiveness of the hypothalamic pituitary-adrenal (HPA) axis to stress (HSR vs. LSR lines).

Replicate selected lines: Two independent (i.e., replicate) lines of rats were bidirectionally selectively bred for differential cumulative ACTH responses to footshock stress. Replicate line breeding was used to discount co-selection artifacts not truly linked to stress reactivity and therefore to validate findings from the first line. This is necessary because coincidental phenotypic associations, present in inbred strains (e.g., Fisher, Lewis rats) and suboptimal selective breeding programs, obscure true genetic co-determination. Lines were originally developed from N/NIH outbred rats obtained from the NIH Animal Genetic Resource. This outbred stock is an 8-way intercross of inbred strains (ACI/N, WKY/N, F344/N, BUF/N, BN/Ssn, WN/N, M520/N, and MR/N) and represents a broad range of laboratory rat genomic differences.

To determine stress responsiveness, cannulae were aseptically inserted into the right jugular vein of young adult (50-60 days) rats under brief anesthesia (isoflurane, 2-3% in oxygen). After surgical recovery, rats were exposed to 0.25 mA footshock sessions for 60 min (0.25 mA, 1-sec duration, 2 shocks/ minute to the paws through a grid floor; Coulbourn HO2-08 grid floor shocker). Three days later the rats received an identical footshock session. Blood was sampled via the catheter for analysis of ACTH levels via a modifed two-site immunoradiometric assay (Allegro kit; Nichols Institute, San Juan Capistrano, CA).. On the morning of the footshock stress experiment, the i.v. cannulas were extended with a 50-cm long catheter filled with heparinized saline. For blood sampling, the volumes withdrawn were 0.3 ml and were replaced with apyrogenic saline, a procedure that does not alter baseline activity of the HPA axis. Integrated ACTH release was assessed using the following

5 time points: basal [unstressed] conditions, and 10, 30, 45 and 60 minutes following the initiation of the footshock. On each of the two experimental shock days, *z*-scores were calculated for each rat according to the position of its ACTH response within the distribution of its gender and generation. Z-scores from the two tests were averaged and breeders were chosen as those with the 12 highest (HSR) and lowest (LSR) average *z*-scores. Footshock testing was initiated 3.5-5.5 hr following the onset of the light cycle to reduce circadian influences.

Several strategies were used to maintain genetic diversity to reduce unintended co-selection of unrelated traits. First, at least 12 breeding pairs were used to form each subline's (e.g., HSR-1) subsequent generation. As shown in **Fig. 1**, even in the absence of selection, genetic diversity is lost when the effective breeding population is too small. Effective breeding populations of 12 pairs retain more of their initial genetic diversity through 18 generations than breeding populations (e.g., 2-3 pairs) that have been used by others for selective breeding (~50% vs. 0.6-4%). The use of 12 breeding pairs per generation also accelerates genetic segregation, or the segregation of coincidental alleles of non-interest from alleles linked to the selected trait. Breeding more than 12 pairs per generation would be very costly with diminishing returns for genetic diversity. Genetic diversity also was preserved during pairing of breeders. Open selection was used whereby extreme scorers were selected from all offspring of the prior generation rather than only from offspring of like-scoring parents. To assign pairs, the trial mating function of Breeder's Assistant Pedigree Software for Fancy Rats (Tenset Tech, Cambridge, England) was used to minimize Wright's coefficient of inbreeding (COI) and the coefficient of relatedness.

Line 1 was in its seventh generation and Line 2 in its fifth generation at the onset of the Addendum. During the funding period, Line 1 advanced to the ninth generation and Line 2 to the seventh generation, representing 4 generations of progress. As shown in **Fig. 2**, the selectively bred lines continue to differ in their ACTH responses to footshock. Figure 2 shows the cumulative ACTH response of female Line 2 HSR and LSR offspring from the S7 generation, the lines which currently differ least in their ACTH response to footshock. As can be seen, approximately 2-fold differences in ACTH response to stress were observed across each stress session. Thus, the HSR and LSR lines remain well-separated in their ACTH response to footshock.

Generalizability of differential HPA responsiveness: To determine whether the differential ACTH shock reactivity of HSR and LSR lines generalized to another stressor and to adrenocortical stress

responses, CORT responses to a brief social defeat were studied in adult male HSR-1 and LSR-1 S7 rats. A resident/intruder procedure was used in which an experimental "intruder" rat implanted with a chronic jugular catheter was placed in the enclosure of a territorial "resident" rat. After submitting, the intruder rat was placed within a wire-mesh cage inside the resident's enclosure through 9 min for continued threat. Blood samples were obtained prior to defeat (basal) and 10, 30 and 60 min post-defeat onset. HSR-1 rats had higher response (+10 min: 312 vs. 194 ng/ml, +30 min: 434 vs. 294 ng/ml), but not basal, CORT levels than LSR-1 rats, resulting in a 53% greater area under the curve during the entire post-stress period ($p<0.05$). The results indicate that the differential HPA-responsiveness of the selectively bred lines also is observed in corticosterone responses and is not specific to the stress on which they were selected. Thus, as desired, the lines differ more generally in their HPA-hormone stress responsiveness.

Outbred controls: N/NIH outbred stock rats (from which the selectively bred lines were derived) were bred randomly in parallel to the selectively bred lines. The offspring act as reference controls for the genetic background and rearing environment of the lines. Fewer breeding pairs (n=6) were used per generation than for selective breeding, as N/NIH rats will be immigrated in the future (n=10 male, 10 female per year) to maintain genetic diversity in the outbred population. This pool of rats also will represent a pool of breeding immigrants in the future to maintain genetic diversity in the selected lines. To select immigrants for selective breeding (n=1 male and female per generation), footshock sessions will be used to identify the extreme scoring members of a generation (lowest/highest 3% of scorers). Four generations of N/NIH outbred rats were bred during the funding period.

Specific Aim 2: To phenotype HSR and LSR sublines with respect to anxiety-, depressive- and other affective spectrum-like behaviors under baseline and social stress conditions.

Anxiety-like behavior: To determine whether HSR and LSR rats also differed in their behavioral responses to stress, which would indicate a general difference in stress reactivity and not only a difference in HPA reactivity, Line 1 offspring were tested for their behavior in the shock-probe test of anxiety (S8 generation). The shock-probe test, also known as the defensive burying test, examined the emission of stress-related active behaviors in the 10 min following receipt of a single shock (1.5 mA). The shock-probe test occurred in a polycarbonate cage that had 2" of wood shavings along the bottom

and a small hole centered in one side 1" above the bedding for the shock-probe. Rats were group-acclimated to the test cage for 45 min without the shock probe present on the two days prior to testing. On the test day, the probe was inserted through the cage hole and connected to a Coulbourn precision shocker (model E13-01), to deliver a 1.5 mA A/C shock when contacted. Rats were tested individually. On shock delivery (<1 sec), verified by a startle response, the probe was deactivated. Bedding was changed and the cage washed between tests. A reliable, treatment-naïve rater scored the emission (bouts) and duration (time) of the following behaviors from video: defensive burying, freezing, grooming, locomotion, rearing, resting, eating/pica, and probe exploration. Stress is known to increase defensive burying, grooming and freezing. Tests occurred from 1000-1300 hr under room lighting. As shown in **Fig. 3**, adult male HSR-1 rats exhibited an anxiogenic-like phenotype, as they exhibited more stress-like behaviors (defensive burying, freezing and grooming), explored the probe less and showed lower levels of non-stress like active behaviors (locomotion, rearing) than did LSR-1 rats. HSR-1 rats showed 2.5-fold more burying, 4.0-fold more grooming and 3.7-fold more freezing, indicating that they did not selectively differ in active vs. inactive anxiogenic-like behaviors. Thus, results of the shock-probe test supported the hypothesis that HSR rats exhibit an anxiogenic-like phenotype.

Line 1 HSR and LSR offspring also were compared in their locomotor responses to a novel environment (S8 generation). The locomotor response to novelty test tested the nocturnal locomotor responses of adult male HSR and LSR rats to being placed within a novel wire-mesh cage or within the same cage on the day following a 3-hr habituation session. The motor activity apparatus consisted of 16 wire mesh cages (20 × 25 × 36 cm), each equipped with two horizontal infrared photocell beams situated along the long axis of the cage, 2 cm above the floor and 16 cm from one another. White noise (70 dB) was present during the first (novel) and second (familiar) test days. As shown in **Fig. 4**, LSR-1 rats exhibited an increased locomotor response to novelty compared to HSR-1 and N/NIH rats (where decreased activity in novel environments may reflect an anxiogenic-like response). Differences were observed as an increase in cage crossings during the first 40 min of the novel test day and only within the first 10 min of the second, familiar test day. Rat lines did not differ in activity levels after acclimation **(Fig. 4)**. Thus, results of the locomotor activity test also suggested an anxiogenic-like phenotype of HSR rats relative to LSR rats, with comparison to the outbred strain suggesting that the LSR line exhibited an anxiolytic-like phenotype relative to the outbred background. Collectively, the

results confirmed that HSR-1 and LSR-1 rats diverged in anxiety-like behaviors not under direct selection.

Ethanol consumption: Stress and anxiety have been associated with increased ethanol intake in humans. This observation has led to the tension-reduction hypothesis of alcoholism wherein excess ethanol consumption is proposed to serve a self-medicating function to relieve dysphoria and symptoms of stress. To determine whether the increased stress responsiveness of HSR rats also was associated with a different proclivity to acquire or maintain ethanol intake, rats were tested for their voluntary intake of sweetened or unsweetened ethanol solutions using the following saccharin-ethanol fading procedure. In all phases of the procedure, rats had *ad libitum* access to a second bottle that contained only water as well as to food:

Voluntary saccharin and ethanol intake: Saccharin-ethanol fading procedure

Daily, nocturnal 2-hr home cage access to a second bottle containing:

Days 1-6	Saccharin (0.2% *w/v*)
Days 7-11	Saccharin + EtOH (5% *w/v*)
Days 12-14	EtOH (5%)
Days 15-17	Saccharin + EtOH (8%)
Day 18	EtOH (8%)
Days 19-21	Saccharin + EtOH (10%)
Day 22	Saccharin (0.1%) + EtOH (10%)
Days 23-24	Saccharin (0.05%) + EtOH (10%)
Days 25-32	EtOH (10%)

As shown in **Figure 5**, male HSR rats drank more of the sweet saccharin solution, of sweetened ethanol solutions (5%, 8% w/v) and of an unsweetened ethanol solution (5%) than LSR rats. Although they did not drink significantly more of the more concentrated ethanol solutions, they did not, on average, drink less of *any* of the 8 solutions that contained ethanol, a frequency unlikely to result from chance alone ($p<0.005$, binomial distribution).

Following acquisition of voluntary ethanol intake, HSR and LSR rats were compared for their ethanol intake after periods of forced abstinence. Lines were compared under two conditions, first after a 5 day deprivation period and then again after a 2 week deprivation period that followed

continuous access to 10% w/v ethanol for 3 weeks. Thus, the forced abstinence schedule was as follows:

Redrinking responses to forced ethanol abstinence:

Days 33-38	5-day abstinence followed by EtOH (10%) access
Days 39-60	Stabilization of 24-hr EtOH access
Days 61-78	2-week abstinence followed by EtOH (10%) access

As shown in **Figure 6**, male HSR rats drank more of the unsweetened 10% w/v ethanol solution than LSR rats after both abstinence periods, exhibiting a greater alcohol deprivation effect than LSR rats. Following the 2-week abstinence period, ethanol intake and preference of HSR rats remained higher than that of LSR rats for at least 3 days. Following re-establishment of stable daily ethanol intake, HSR rats continued to show significantly ($p<0.05$) greater intake of and preference for 10% w/v ethanol than LSR rats. Each day HSR rats drank 2.5$\pm$0.3 g/kg vs. 1.6$\pm$0.4 g/kg of ethanol for LSR rats with preference ratios of 59$\pm$6 and 36$\pm$10%, respectively.

Body weight: Stress also has been associated with overeating and overweight in humans, and is a putative risk factor for visceral obesity. Given that HSR rats overconsumed a sweetened saccharin solution, it also was of interest to compare their body weights to that of LSR rats across the first year of life. As shown in Figure 7, male HSR rats were significantly heavier than LSR rats beginning from young adulthood. Weight of LSR rats was similar to that of N/NIH outbred stock rats, indicating that high stress responsive rats had diverged on this trait. Weight differences were observed in three different cohorts of rats drawn from two different generations, supporting the robustness of the effect.

In summary, initial phenotyping has shown that:

- HSR-1 rats exhibit an anxiogenic-like phenotype relative to LSR-1 rats in the shock-probe test and in their locomotor responses to novelty.

- HSR-1 males are heavier than LSR-1 and outbred N/NIH males throughout adulthood.

- HSR-1 males exhibited increased intake of a palatable saccharin solution and sweetened and unsweetened ethanol solutions relative to LSR-1 males.

- Following forced abstinence, HSR-1 males exhibited greater levels of unsweetened ethanol intake and ethanol preference than did LSR-1 males.

KEY RESEARCH ACCOMPLISHMENTS

- Replicate lines that differ in their ACTH responses to stress (high stress responsive [HSR] and low stress responsive [LSR] were advanced two generations each (total of 4 generations). Lines remain separated in their ACTH responses to footshock, as intended.

- A reference pool of outbred N/NIH rats were random-bred in parallel to the HSR and LSR lines. The N/NIH rats represent an appropriate control group to which HSR and LSR rats can be compared as they share the same genetic background and rearing environment.

- The differential HPA-axis responsiveness of HSR and LSR rats was shown to generalize to corticosterone responses to a stressor other than was used for selective breeding.

- HSR rats were found to be heavier from young adulthood through at least one year of life.

- HSR rats were found to overconsume a sweetened solution almost 2-fold.

- HSR rats were found to acquire greater voluntary intake of sweetened and unsweetened ethanol. HSR rats also exhibited a greater alcohol deprivation effect after 5 and 14 days of forced abstinence than LSR rats. At the completion of all tests, HSR rats drank more ethanol and showed greater preference for ethanol than LSR rats.

- HSR rats were found to exhibit an anxiogenic-like phenotype under stressful conditions, as reflected in the shock probe test and in reduced activity in an unfamiliar environment.

REPORTABLE OUTCOMES:

A manuscript reporting results of the project was published during the funding period:
Izzo E, Sanna PP, Koob GF. Impairment of dopaminergic system function after chronic treatment with corticotropin-releasing factor. *Pharmacology, Biochemistry and Behavior* (2005) 81:701-8.

Two additional manuscripts that report results from the earlier studies of social defeat as well as of the development and current phenotyping of the replicate rat lines bred are in preparation.

The following presentation was made at the Society for Neuroscience conference:
Zorrilla EP, Rivier CL, Roberts AJ, Koob GF. Rats selectively bred for high stress responsiveness exhibit greater body weight and increased saccharin and ethanol intake Program No. 580.11. 2004 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.

The following two grant applications were made to the National Institutes of Health to pursue the present findings further:
Heritable differences in stress reactivity and dysphoria
National Institute of Mental Health: 1 R21 MH077096-01
PI: Eric Zorrilla, Co-PI: George Koob

Coheritability of stress reactivity and obesity: Role of diet composition
National Institute of Digestive, Diabetes and Kidney Disorders: 1 R21 DK074865-01
PI: Eric Zorrilla, Co-PI: George Koob

Finally, the rat lines will be a major resource for the scientific community which can be developed further and disseminated widely.

CONCLUSIONS:

Collectively, the results indicate that ACTH responsiveness to footshock stress is heritable, is a generalizable trait that also alters corticosterone responsiveness to other stressors, and is amenable to

selective breeding in the rat. Genetic differences in stress responsiveness in replicate line 1 were associated with differences in anxiety-like behavior, body weight gain and voluntary intake of sweet solutions and ethanol -- possible indications of altered hedonic homeostasis. For some traits, the high stress responsive line was found to diverge from outbred stock, whereas for other traits the low stress responsive line was found to be different. Studies of replicate line 2 will follow. The replicate lines may help identify the genetic basis of hyper- and hypo-stress responsiveness and associated stress-related disorders.

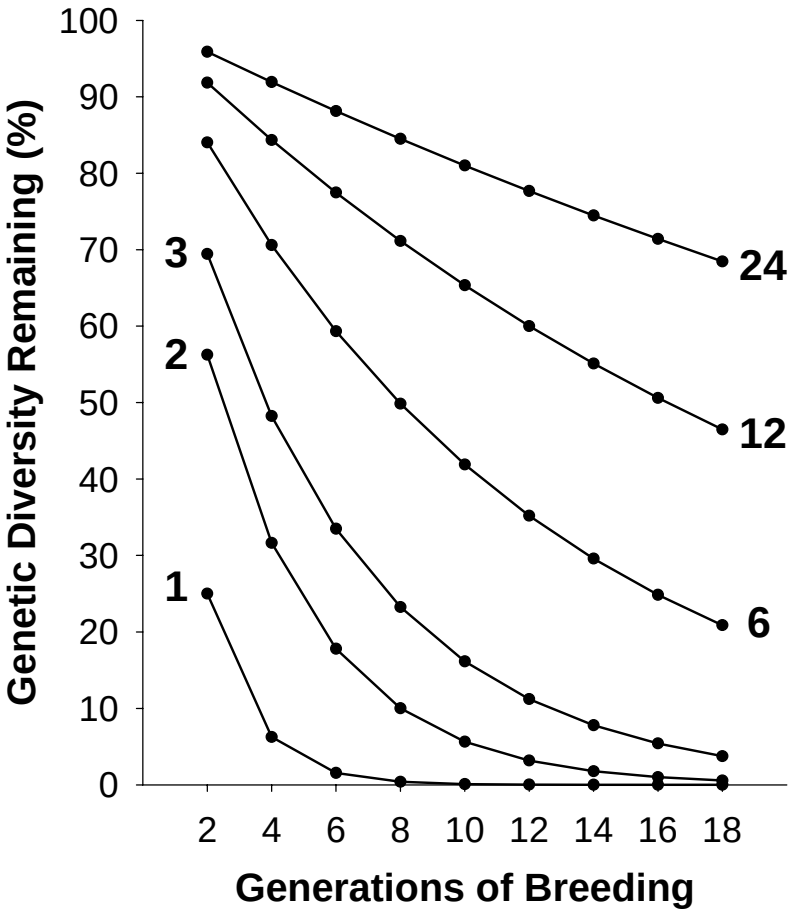

Fig. 1. Loss of genetic diversity as a function of the effective breeding population size. The number of breeding pairs used per generation is shown numerically to the side of its respective diversity function. The current breeding program uses at least 12 breeding pairs per generation, balancing resource costs vs. the loss of genetic diversity.

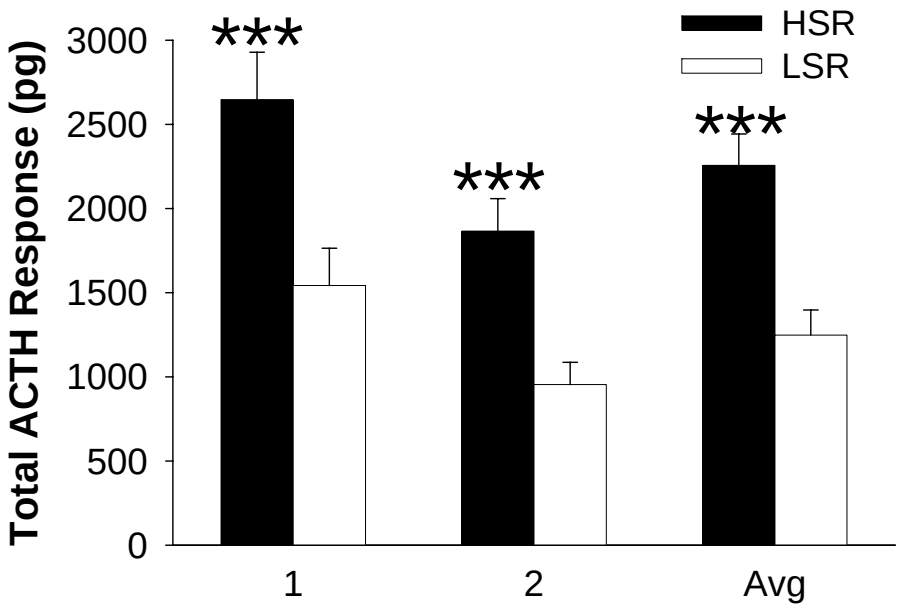

Figure 2. Mean (± SEM) total ACTH responses (pg) during two 60-min 0.25 mA footshock sessions in female high-stress responsive (HSR) and low-stress responsive (LSR) S7 generation rats from replicate line 2. Consistent with their intended phenotype, female (*n*'s=20-25 per subline) HSR offspring rats exhibited greater cumulative ACTH responses to footshock than their LSR counterparts (***$p<0.0001$ vs LSR, Student's *t*-test).

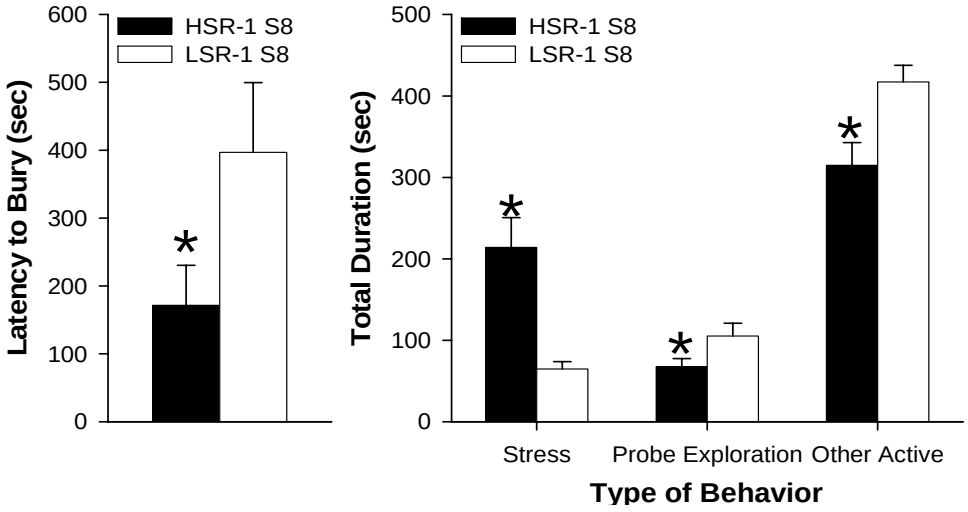

Figure 3. Mean (+ SEM) latency to bury (left panel) and duration of different behaviors (right) in the 10-min following receipt of a 1.5 mA shock in the shock-probe test. HSR rats exhibited stress-related behaviors (burying, freezing, grooming) earlier and longer than did LSR rats. HSR rats explored the probe less and showed less of other active behaviors (*$p<0.05$ vs LSR, Student's *t*-test).

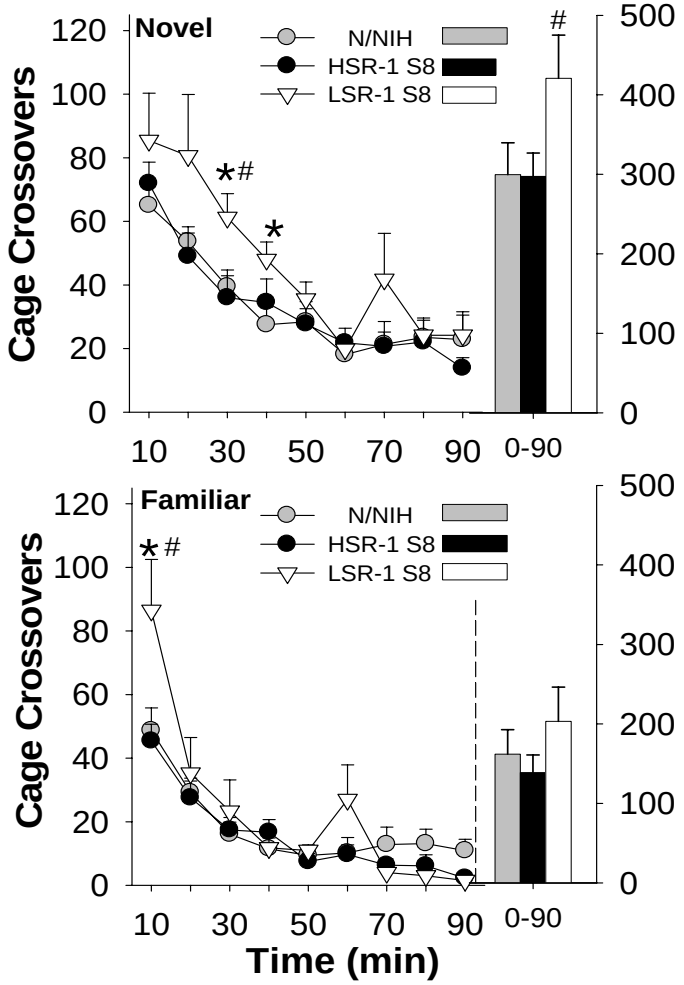

Figure 4. Mean (+ SEM) locomotor activity in a novel (top) or familiar (bottom) photocell test cage in HSR-1 and LSR-1 rats of the S8 generation and outbred N/NIH rats. (*p<0.05 vs N/NIH outbred stock, #p<0.05 vs HSR line, Student's t-test).

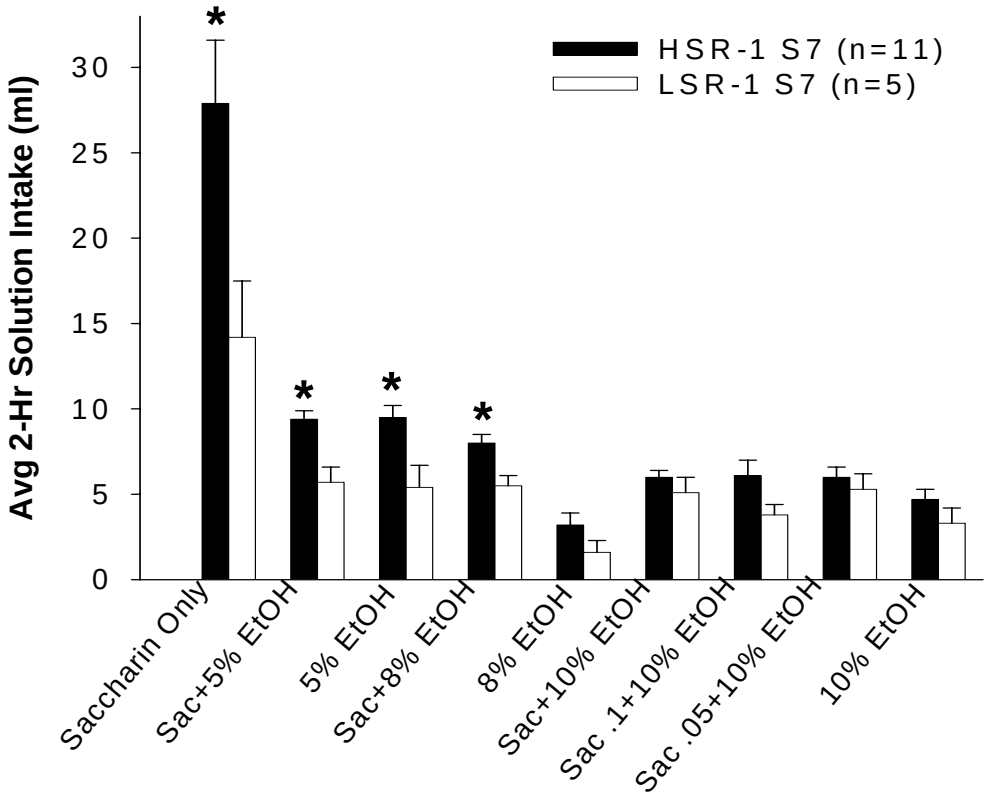

Figure 5. Mean (+ SEM) acquisition of intake of sweetened (saccharin only), sweetened ethanol (saccharin+ethanol) or ethanol (ethanol only) solutions in Line 1 male high stress responsive (HSR-1) and low stress responsive (LSR-1) rats of the S8 generation during a saccharin->ethanol fading procedure. Each bar represents the average from 1-6 days of nocturnal, limited access (2 hr) sessions. Concentrations are expressed as *w/v*. *$p<0.05$ vs. LSR line (Student's *t*-test).

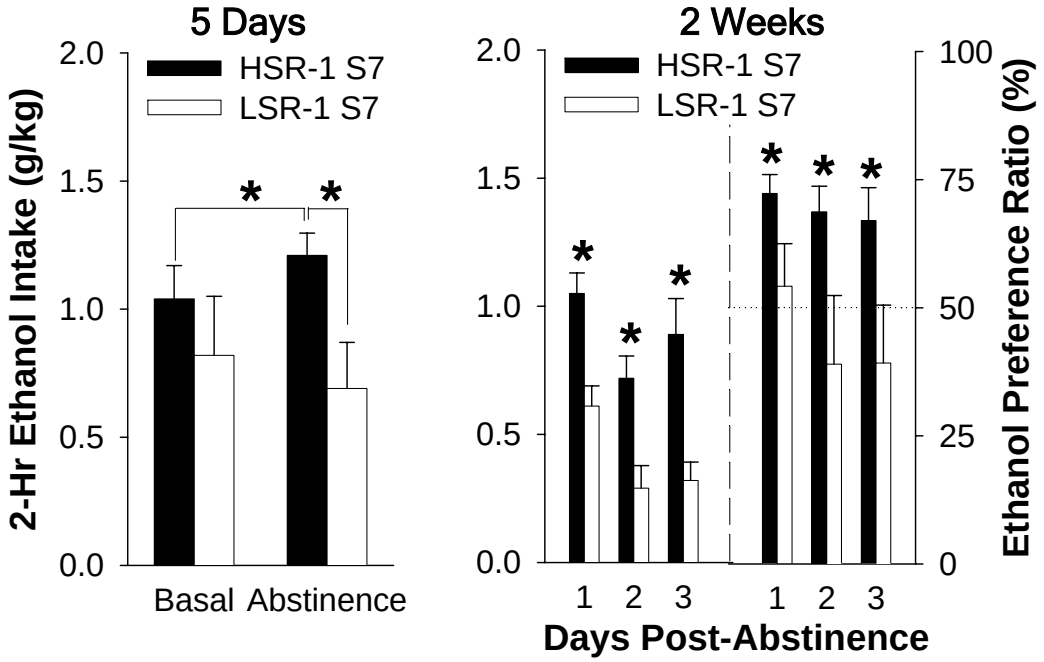

Figure 6. Effects of 5 days (left panel) or 2 weeks (right) of forced abstinence from ethanol on subsequent voluntary ethanol (10% *w/v*) intake of Line 1 male HSR and LSR rats of the S8 generation during the first 2 hours of daily nocturnal access. HSR rats drank significantly more ethanol than LSR rats following brief or extended deprivation periods. Following the 2 week abstinence period, HSR rats continued to show greater intake of and preference for ethanol for at least 3 days. Rats had *ad libitum* access to food and water at all times during testing. *$p<0.05$ vs. LSR line (Student's *t*-test).

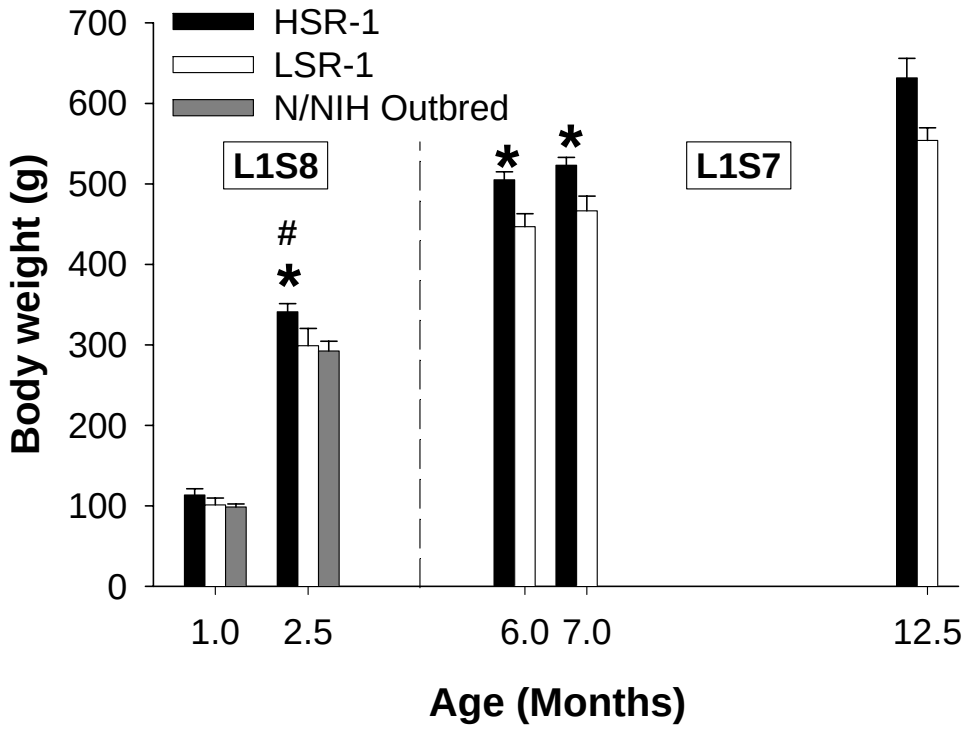

Figure 7. Mean (+SEM) body weight of Line 1 high stress responsive (HSR-1), low stress responsive (LSR-1) and outbred N/NIH rats across the first year of life. HSR-1 rats are significantly heavier than LSR-1 rats beginning from young adulthood, with LSR-1 rats showing body weight similar to that of N/NIH outbred rats. Differences were observed in two different groups of S7 generation rats (6-7 months and 12.5 months) as well as in a group of S8 generation rats (2.5 months). *$p<0.05$ vs. LSR line, #$p<0.05$ vs. N/NIH outbred stock (Student's *t*-test).